Cell Density and Culture Factors Regulate Keratinocyte Commitment to Differentiation and Expression of Suprabasal K1/K10 Keratins

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Irreversible growth arrest and commitment to differentiation are among the earliest events in the program of cellular terminal differentiation. The transition from highly proliferative human keratinocytes in subconfluent culture to stationary cells in confluent culture was studied in a serum-free culture system to identify conditions that regulate the initiation of terminal differentiation in keratinocytes. We observed that culture confluence strongly induced commitment to terminal differentiation, as demonstrated by a dramatic loss of keratinocyte clonogenicity. Commitment was accompanied by the rapid induction of early differentiation markers, represented by expression of suprabasal keratin 1 (K1) and 10 (K10) genes. Induction of differentiation was independent of low (0.1 mM) or high (1.5 mM) calcium concentration in the medium. Epidermal growth factor suppressed expression of K1 and K10 mRNA in cultures induced to differentiate. Suspension of keratinocytes in methylcellulose medium failed to induce in subconfluent cultures, or enhance in confluent cultures, the expression of K1 and K10 genes. Subconfluent cells cultured in medium containing high calcium and no exogenous growth factor induced expression of K1 and K10 transcripts, but commitment and loss of proliferative potential were not observed. Confluent cell density primarily controlled keratinocyte commitment to terminal differentiation and differentiated keratin gene expression. However, suprabasal K1 and K10 gene expression also was regulated by medium calcium and exogenous growth-factor concentrations in subconfluent cultures that promoted cell-cell association. Epidermal growth factor inhibited the expression of suprabasal keratins but not the commitment to terminal differentiation mediated by cell confluence. Control of keratinocyte commitment and expression of selected differentiation genes are mediated by cell confluence and, at subconfluence, by specific culture factors. *Key words: calcium/cell density. J Invest Dermatol 104:271–276, 1995*

The epidermis is a stratified squamous epithelium with the main cell type, keratinocytes, superpositioned and organized into multiple discrete layers. Keratinocytes migrate outwardly from the proliferating, basement-membrane–anchored basal layer to the spinous, granular, and finally cornified layers. During this directional traverse within the epidermis, keratinocytes undergo a complex program of terminal differentiation, also called keratinization (reviewed in [1]).

As part of this maturational program, basal keratinocytes, expressing keratins 5 (K5) and 14 (K14), depart from their residence apposing the basal lamina and venture to a suprabasal location where cells rapidly induce synthesis of the differentiation-specific keratins 1 (K1) and 10 (K10). In concert with migration from the basal layer and expression of differentiated keratin genes, keratinocytes also regulate the expression of many other genes that are linked to terminal differentiation and development of an intact epithelium.

Differentiating epidermal keratinocyte populations arise from clonogenic stem cells that produce rapidly replicating, transit-amplifying keratinocytes [2,3]. Proliferative keratinocytes can be isolated from the epidermis and propagated under serum-free or defined culture conditions *in vitro* [4,5]. Complex and undefined cellular events convert proliferative keratinocytes to irreversibly growth-arrested cells that become committed to terminal differentiation [5,6]. Finally, committed keratinocytes can be induced to express markers of terminal differentiation [6–8].

The precise mechanisms that regulate growth of the proliferative cell population, trigger keratinocyte commitment and control epidermal maturation still are largely undefined. Evidence suggests that not only proliferative keratinocytes, but also those cells committed to terminal differentiation, occupy the basal layer [3,9,10]. In a resting, unperturbed state, the epidermis appears to be largely self-sufficient, including the capacity to coordinate keratinocyte growth and differentiation.

Requirements for keratinocyte growth control include the expression and interaction of cell-adhesion molecules (especially of the integrin family) with extracellular matrix molecules [10,11], expression and reception of growth factors that potentiate (i.e., epidermal growth factor [EGF], transforming growth factor-α, amphiregulin) [12,13] or inhibit (i.e., transforming growth factor-β) [14] proliferation of keratinocytes, regulation of extracellular and intracellular calcium levels that may initiate or facilitate terminal differentiation [15–18], and regulation of the ambient

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supply of various essential nutrients such as vitamins A and D [8,19], among many other factors.

Human epidermal keratinocytes propagated in a serum-free culture system based on the basal medium formulation MCDB 153 require exogenous growth factors to initiate cell growth. Once keratinocyte colonies are established, exogenous growth factors are no longer required, and cells exhibit autonomous proliferation [13]. Highly clonogenic populations of keratinocytes cultivated at subconfluent cell densities decrease dramatically when keratinocytes reach confluence in culture [5]. Selected culture conditions such as transforming growth factor-β treatment or nutrient arrest induce reversible growth arrest of keratinocytes [14,20]. Other growth-arrest states, including growth to confluence or suspension culture, are essentially irreversible as determined by clonal growth and cell cycle analysis [5,20].

The present studies were conducted to examine further and reevaluate early events in the cellular control of proliferation, reversible or irreversible growth arrest, commitment to differentiation, and terminal differentiation, which constitute the main program of growth and differentiation for the epidermal keratinocyte. We examined keratin gene expression, including K14 as the basal proliferative cell marker and K1 and K10 as among the earliest representative markers of terminal differentiation that are expressed by committed basal and suprabasal keratinocytes. The results of these investigations demonstrate that complex interactions between cell density, growth-factor presence, and medium calcium concentrations cooperate in culture. Together, these factors mediate irreversible growth arrest and expression of the differentiation-specific keratin genes K1 and K10. We also present evidence that elevated medium calcium concentration is neither required nor sufficient to induce keratinocyte commitment and terminal differentiation and that, under some conditions, K1 and K10 gene expression is induced without loss of keratinocyte clonogenicity.

MATERIALS AND METHODS

Culture of Normal Human Keratinocytes Human keratinocytes were isolated from neonatal foreskin specimens, and primary cultures were initiated and maintained in a replicative state with MCDB 153 medium supplemented with 0.2% (v/v) bovine pituitary extract, EGF (10 ng/ml), insulin (5 μg/ml), hydrocortisone (5×10^{-7} M), ethanolamine (1×10^{-4} M), and phosphoethanolamine (1×10^{-4} M) [5]. This medium is designated "complete medium." "Standard medium" is complete medium without the growth factors, hormones, and additives of EGF, insulin, and bovine pituitary extract. The standard-medium calcium concentration was 0.1 mM and is designated "low" calcium medium. "High" calcium medium was prepared by adding $CaCl_2$ solution to a final concentration of 1.5 mM. Subconfluent keratinocytes from primary cultures were plated into secondary culture at $1–10 \times 10^3$/cells/cm^2. Cultures were washed repeatedly with standard medium when switched from complete to standard medium. Clonal growth assays were performed as described previously [5].

For suspension in semi-solid medium, keratinocytes were trypsinized, resuspended in a small volume of medium and then added to 1.5% (w/v) methylcellulose-containing standard medium. In selected experiments, EGF (10 ng/ml) or $CaCl_2$ (1.5 mM) was added to the suspension medium.

RNA Isolation and Northern Blot Analysis Poly(A)RNA was isolated from keratinocytes cultures by the method of Schwab *et al* [21]. After resolution on 1.2% agarose-formaldehyde gels and transfer, the membranes were hybridized at 43°C with randomly primed DNA probes labelled with [α-^{32}P]dCTP [22]. The probes included cDNAs specific for the human K1 or K10 suprabasal keratins or specific for the K14 basal keratin [23]. High-stringency washes (0.1 × sodium citrate/sodium chloride buffer/0.1% sodium dodecylsulfate at 65°C) were applied to the hybridized membranes before autoradiographic exposure. Each membrane also was hybridized with an identically labeled 1B15 cDNA probe for the constitutively expressed cyclophilin gene [24] to assure equivalent loading and transfer of RNA. Densitometric analysis was performed on a Shimadzu CS9000U densitometer.

Immunofluorescent Staining of Keratins Human keratinocytes grown on glass coverslips were fixed in methanol:acetone (1:1) solution. The cells were rehydrated, blocked with 1% bovine serum albumin/5% bovine serum, rinsed, and incubated with an affinity-purified rabbit antibody to K10 or a similar antibody to K14 [23]. After washing, a fluorescein-isothiocyanate–conjugated goat anti-rabbit antibody (Southern Biotechnology Birmingham, AL) was applied. Cells were mounted in p-phenylenediamine-90% glycerol solution and photographed using a Zeiss Axiophot microscope. No specific immunoreactivity was found when the primary antibodies were omitted.

Western Blot Analysis Cell lysates were prepared by scraping cultures into sample buffer, incubating at 100°C for 5 min, and centrifuging at high speed. The supernatant fractions were recovered and separated electrophoretically by 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis. The proteins were electro-transferred to Immobilon-P membranes (Millipore, Bedford, MA), and the membranes were blocked overnight in 5% bovine serum albumin. K1 and K14 proteins were detected by incubation for 2 h with affinity-purified sheep antibodies [23], followed by three 10-min washes and 1 h incubation with a peroxidase-conjugated donkey anti-sheep antibody (Sigma, St Louis, MO). The keratin bands were visualized using the ECL detection system (Amersham, Arlington Heights, IL) and then positioned relative to molecular-weight markers by amido-black staining (Sigma) of membranes.

RESULTS

Clonogenic Potential of Subconfluent Versus Confluent Keratinocyte Cultures Clonal growth assays of keratinocytes cultivated in complete **(Fig 1*A,B*)** or standard **(Fig 1*C,D*)** medium containing either low (0.1 mM, **Fig 1*I***) or high (1.5 mM, **Fig 1*II***) calcium concentrations showed that the clonogenic potential of keratinocytes decreased dramatically when cultures became confluent and converted their proliferative rate from an exponential growth phase to a stationary or plateau growth phase. Proliferation of keratinocytes at higher cell density proceeded in the absence of exogenous growth factors, which we termed autonomous growth [13]. The clonogenic potential of autonomously proliferating cells was sustained at low or high medium calcium concentration **(Fig 1*C*)** and, as in cultures propagated in medium containing exogenous growth factors, decreased significantly when confluence was attained **(Fig 1*D*)**. In addition, the results show that the extracellular calcium concentration of the culture medium exerted no significant effect on the clonogenic potential of subconfluent or confluent cultures. These findings indicate that the large majority of proliferative keratinocytes were committed rapidly to irreversible growth arrest at cell confluence.

Confluent Keratinocyte Cultures Express K1 and K10 Suprabasal Keratins We determined whether this initial step in keratinocyte commitment induces the expression of suprabasal

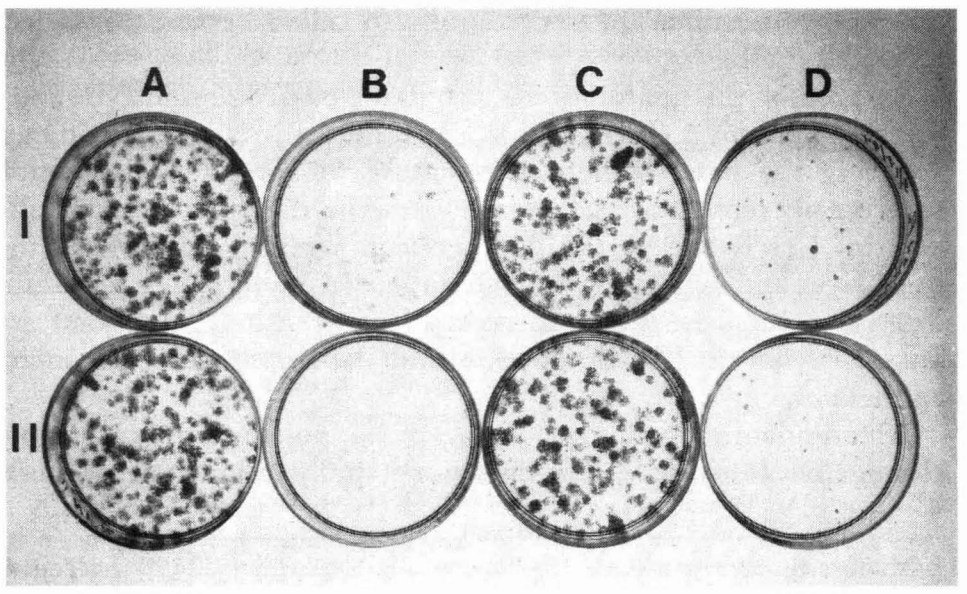

Figure 1. Confluence regulates clonogenic potential of human keratinocytes. Keratinocytes were cultured in complete medium (*A,B*) or in standard medium (*C,D*), containing either 0.1 mM (low) Ca^{++} (*I*) or 1.5 mM (high) Ca^{++} (*II*). Cells grown in standard medium had been switched from complete medium 48 h before cell harvest for clonal assay. Human keratinocytes were proliferating exponentially in subconfluent culture (*A,C*) or were at stationary phase of growth in confluent culture for 24 h (*B,D*). Cells were trypsinized, washed, and replated at a clonal density of 500 cells/60-mm dish in complete MCDB 153 medium. After 10 d, culture plates were fixed, stained, and photographed.

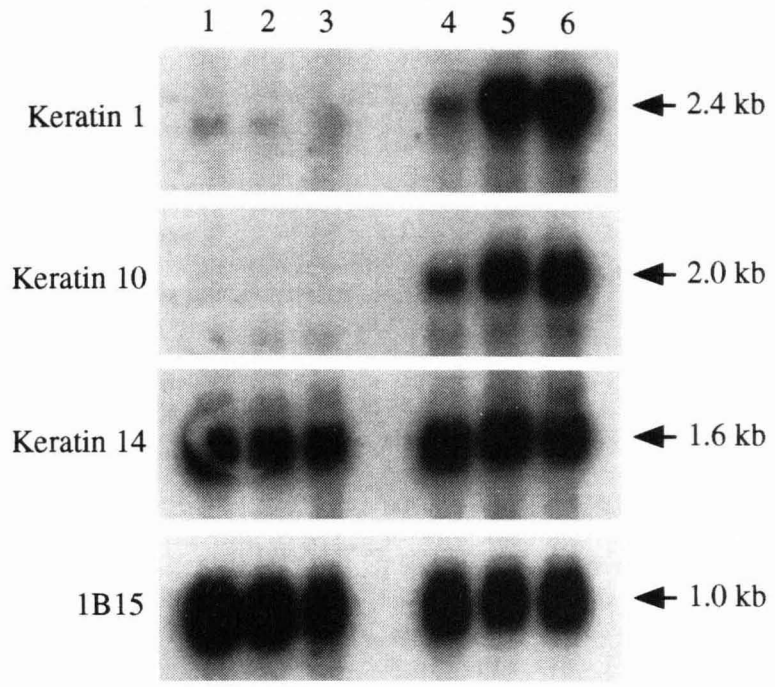

Figure 2. K1 and K10 mRNA expression in confluent keratinocyte cultures. Cells were plated at 5×10^3/cells/cm^2 and cultured in complete medium. At approximately 20% (*lanes 1–3*) or 90% (*lanes 4–6*) of confluence, cultures were maintained in complete, medium with 0.1 mM Ca^{++} (*lanes 1 and 4*), switched to standard medium with 0.1 mM Ca^{++} (*lanes 2 and 5*) or switched to standard medium with 1.5 mM Ca^{++} (*lanes 3 and 6*). Poly(A)RNA was harvested from the cultures 2 d later, at approximately 80% to 90% of confluence (*lanes 1–3*), or total confluence after at least 24 h (*lanes 4–6*). Five micrograms of each sample was used for Northern blot hybridization with probes specific for K1, K10, K14, or 1B15 transcripts.

keratin markers K1 and K10, which are linked to terminal differentiation. Poly(A)RNA was harvested from subconfluent (80% to 90% cell confluence, as assessed directly by phase contrast microscopy) or from fully confluent keratinocyte cultures. Where indicated, exogenous growth factors were removed and the medium calcium concentration was elevated to 1.5 mM for 48 h before RNA isolation. Northern analysis **(Fig 2)** shows that confluence of keratinocyte cultures rapidly and strongly induced mRNA expression of the K1 and K10 differentiation-specific keratin genes. Relative levels of expression at confluence of the suprabasal keratins compared to 1B15 were calculated by densitometric analysis. The presence of exogenous growth factors (*lane 4* compared to *lane 5*) reduced the steady-state level of K1 and K10 transcripts by 92% and 75%, respectively. High (1.5 mM) medium calcium concentration at confluence did not significantly alter K1 and K10 gene expression. Rapidly growing, subconfluent cultures of keratinocytes, grown in the presence or absence of exogenous growth factors and 0.1 mM medium calcium did not express K1 or K10 mRNA. However subconfluent, growth-factor–deficient cultures exposed to high-calcium medium expressed very low levels of K1 and K10 transcripts as revealed by prolonged blot exposure (data not shown). K14 mRNA expression increased, but not dramatically, under confluent conditions.

Accumulation of keratin proteins confirms that K1 and K14 gene expression is regulated primarily at the level of transcription. **Figure 3*a*** shows total protein staining of the blotting membrane and demonstrates that K1- and K10-corresponding bands were identified in confluent cultures grown in the absence of exogenous growth factors (*lane 7*). **Figure 3*b*** shows K1 protein by immunodetection and its progressive accumulation in confluent keratinocyte cultures propagated in the absence of growth factors. Additional bands of lower molecular weights also were detected by this antibody when K1 accumulated in culture over time. These species also have been reported by others [25] and may correspond to a degradation product of K1. By comparison, K14 protein content was fairly constant for every culture condition examined **(Fig 3*c*)**.

Immunofluorescent microscopy demonstrated that suprabasal K10-expressing cells were located singly or as small clusters and were distributed focally but uniformly over the confluent cultures maintained in the absence of exogenous growth factors **(Fig 4*b*)**. The morphology of K10-expressing cells was larger and angulated compared to the majority of smaller, basal located, K10-negative keratinocytes. Exogenous growth-factor–containing medium suppressed the expression of K1 protein (**Fig 3*b***, *lane 4*), as well as the detection of K10-positive keratinocytes **(Fig 4*a*)**, under identical culture conditions. Subconfluent cultures grown in the presence or absence of exogenous growth factors at low calcium concentration failed to express any suprabasal keratin protein in cell extracts (**Fig 3*b***, *lanes 1 and 2*). Extracts from subconfluent cultures grown in medium containing high (1.5 mM) calcium concentration and no exogenous growth factors (standard medium) revealed modest accumulation of K1 (**Fig 3*b***, *lane 3*; see also **Fig 7**), which paralleled the very low level of K1 mRNA expression.

The roles of cell density and the duration of confluency versus the duration in culture on the induction of K1 and K10 mRNA expression were examined further in keratinocyte cultures plated at two different densities (1×10^3 or 5×10^3 cells/cm^2) and then switched simultaneously to growth-factor–deficient, standard medium. Two days later, when the higher-density cultures reached approximately 90% confluence, poly(A)RNA was harvested from both low- and high-density cultures. This was repeated again 2 and 4 d later **(Fig 5)**. Subconfluent cultures did not express K1 mRNA (**Fig 5**, *lanes 1–3*) or K10 mRNA (data not shown). The higher-density cultures attained confluence (within 24 h of extraction) before the lower-density cultures and induced expression of K1 (**Fig 5**, *lane 4*) and K10 (data not shown) mRNA. Eventually, after 4 d, the low–cell-density culture had reached confluence (within 24 h of extraction), and both cultures initiated at low or higher cell density expressed the suprabasal K1 and K10 transcripts (**Fig 5**, *lanes 5,6*). Over the culture period of this experiment, the expression of K14 remained relatively constant, indicating that whereas an increasing proportion of cells became committed to differentiate and express K1 and K10 mRNA, a fairly constant, largely basal population of keratinocytes continued to express the K14 phenotype.

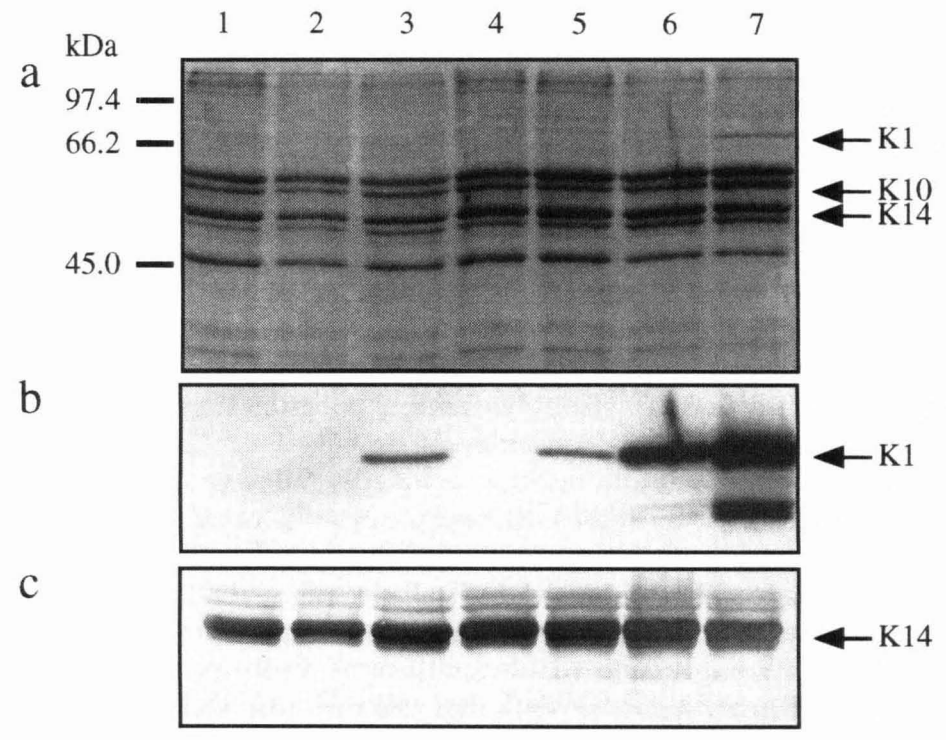

Figure 3. K1 and K14 protein expression in subconfluent or confluent keratinocyte cultures. Cellular protein extracts were prepared from subconfluent (*lanes 1–3*) or confluent (*lanes 4–7*) cultures cultivated for 2 d with EGF and 0.1 mM Ca^{++} (*lanes 1 and 4*), without growth factors and with 0.1 mM Ca^{++} (*lanes 2 and 5*), without growth factors and with 1.5 mM Ca^{++} (*lane 3*), or without growth factors and with 0.1 mM Ca^{+++} for 4 d (*lane 6*) or 6 d (*lane 7*). Positions of molecular weight markers are indicated on the left (*a*) and *arrows* identify the positions of K1, K10, and K14. *a*) Amidoblack total protein staining. *b*) Immunodetection of K1 on the blot shown on panel *a*. *c*) Immunodetection of K14 on a blot run simultaneously with identical samples.

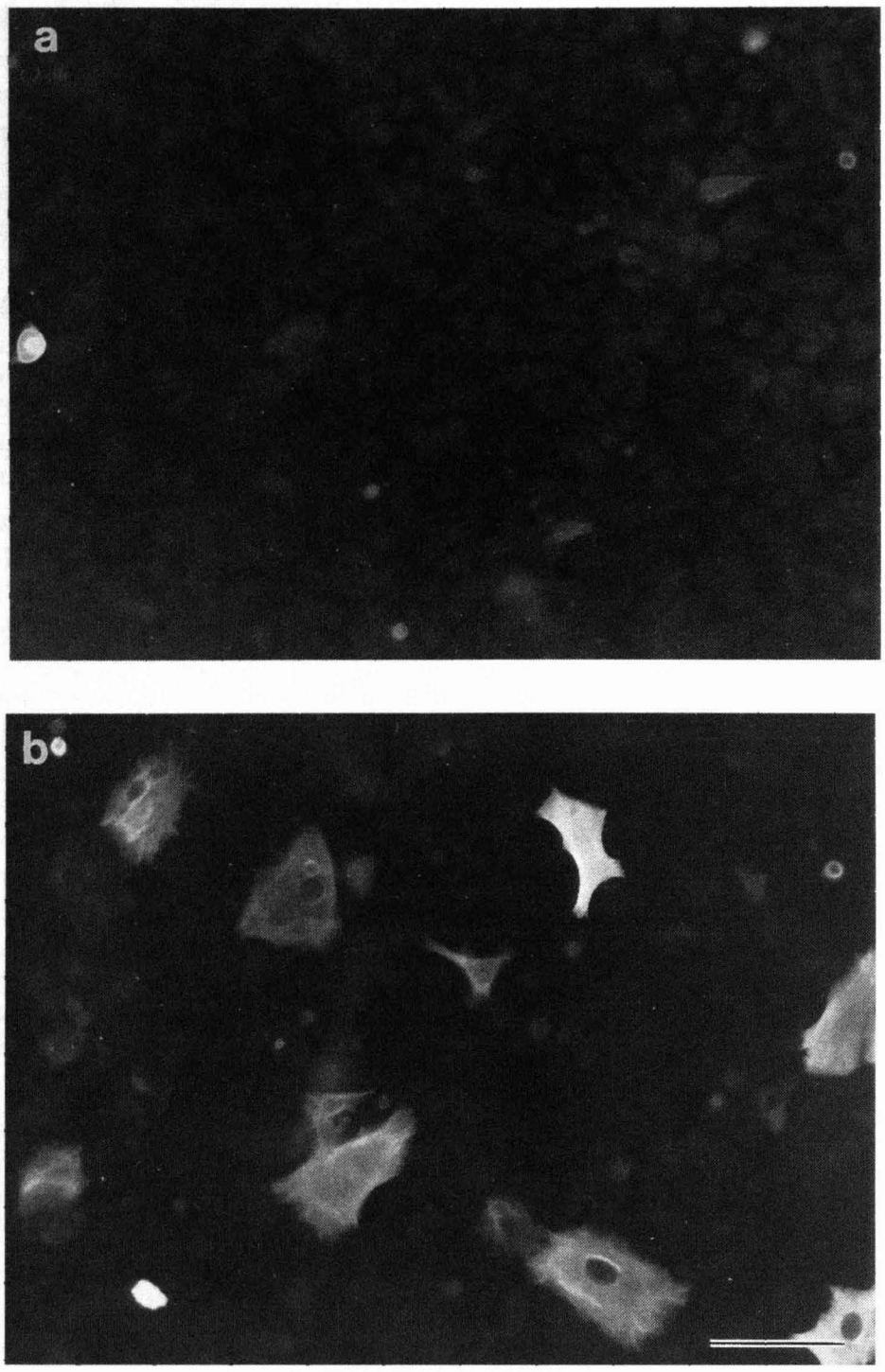

Figure 4. K10 protein expression in confluent keratinocyte cultures. Keratinocytes were grown to confluence in complete medium (*a*) or grown for 2 d in standard medium (*b*) before fixation and indirect fluorescein isothiocyanate-staining of keratin 10. *Bar*, 100 μm.

Suspension Culture Does Not Induce or Increase K1 and K10 mRNA Expression Investigators have used suspension culture to induce irreversible growth arrest and to markedly promote and accelerate terminal differentiation [10,20,25,26]. We anticipated that suspension culture of subconfluent or confluent keratinocytes would strongly induce or enhance K1 and K10 mRNA expression and down-regulate levels of K14 transcripts. Surprisingly, suspension culture repeatedly failed to induce expression of K1 or K10 mRNA in keratinocytes derived from subconfluent cultures grown for at least 2 d in the absence of exogenous growth factor (**Fig 6**, *lanes 1–3*). Even high (1.5 mM) calcium concentration in the suspension culture was unable to trigger K1 or K10 mRNA expression (data not shown). Only confluent keratinocytes of growth-factor–deficient cultures that already had been induced to express K1 and K10 mRNA continued to express steady-state levels of K1 and K10 transcripts in suspension culture (**Fig 6**, *lanes 5–9*). However, K1 and K10 mRNA levels (**Fig 6**, *lane 4*) were not enhanced during this period of suspension. After 24 h of suspension, relative levels of K1 and K10 mRNA were decreased, especially when EGF was present (**Fig 6**, *lane 9*). The levels of K14 transcript also decreased, suggesting that the basal cell phenotype was progressively lost in suspension culture over time.

We have shown previously that keratinocytes lose clonogenicity within 8 h of methylcellulose suspension culture [20]. Similarly, treatment of subconfluent keratinocyte cultures with the active

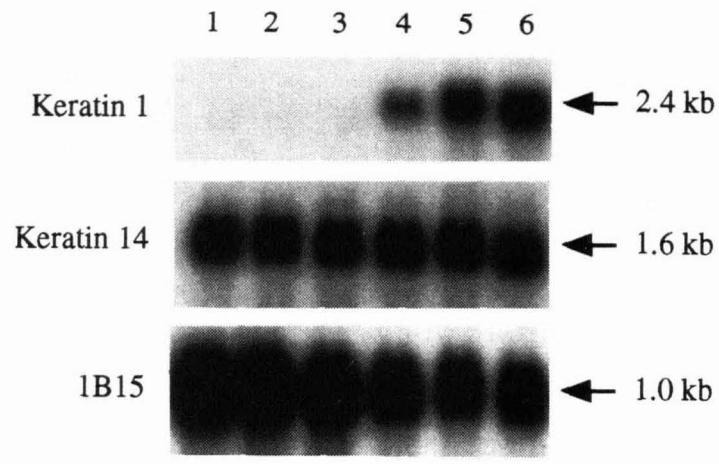

Figure 5. K1 mRNA expression: dependence on cell density and culture confluence. K1 or K14 transcripts and 1B15 transcripts were probed in poly(A)RNA preparations isolated from cultures initiated simultaneously and plated at 1×10^3 (*lanes 1, 3, and 5*) or 5×10^3 (*lanes 2, 4, and 6*) cells/cm^2 and cultivated for 2 d (*lanes 1 and 2*), 4 d (*lanes 3 and 4*) or 6 d (*lanes 5 and 6*) in standard medium before RNA extraction. Samples (5 μg) were subjected to Northern analysis by hybridization with the specific probes.

phorbol ester, 12-O-tetradecanoylphorbol-13-acetate, strongly induced irreversible growth arrest and commitment to terminal differentiation [27]. Subconfluent, growth-factor–deficient cultures exposed to 12-O-tetradecanoylphorbol-13-acetate (10 ng/ml) also failed to express K1 or K10 transcripts, as detected by Northern analysis (data not shown).

These results clearly demonstrate that expression of K1 and K10 genes is induced selectively in irreversibly growth-arrested cultures at confluence, regardless of the extracellular calcium concentration. Further, suspension- or phorbol-ester–mediated loss of clonogenicity is not sufficient for suprabasal keratin mRNA expression. However, we show in the following experiments that expression of K1 and K10 genes in keratinocytes is not inherently linked to loss of clonogenic potential and commitment to terminal differentiation.

Cellular Control of K1/K10 Expression: Effect of Cell Density and High Calcium Concentration Subconfluent cultures switched to growth-factor–deficient (standard) medium containing high (1.5 mM) calcium concentration expressed K1 protein (**Fig 3**, *lane 3*). By phase contrast microscopy, smaller colonies of low-density cultures initiated at 1×10^3 cells/cm^2 exhibited few mitotic cells, whereas cultures initiated at higher cell densities (5×10^3 cells/cm^2) and switched to the same growth-factor–deficient, high-calcium culture medium contained larger colonies with many

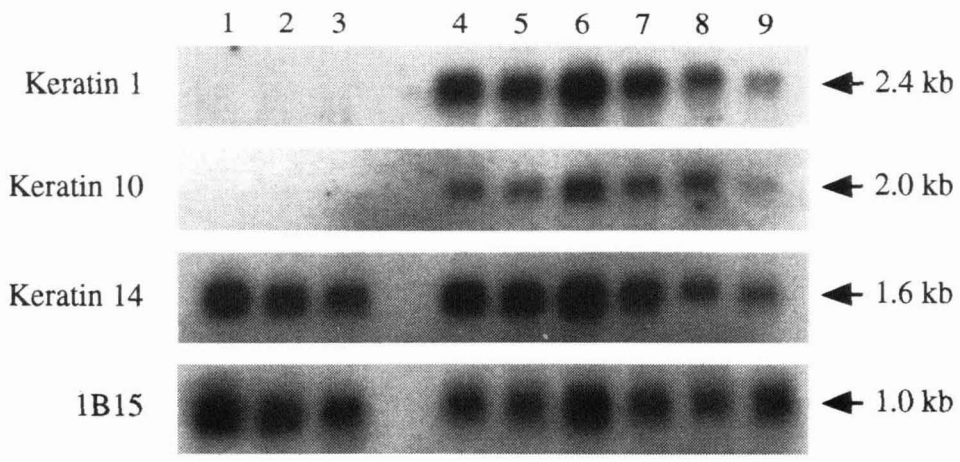

Figure 6. Basal (K14) or suprabasal (K1 and K10) keratin mRNA expression in suspension culture of keratinocytes. Cells were collected from subconfluent cultures grown for 2 d in standard, 0.1 mM Ca^{++} medium and suspended in standard medium containing 1.5% methylcellulose (*lanes 1–3*) or from confluent culture treated identically (*lanes 4–9*). In *lanes 7 and 9*, EGF (10 ng/ml) was added to the suspension medium. Poly(A)RNA was harvested from cultures immediately after trypsinization (*lanes 1 and 4*), or after 4 h (*lane 5*), 8 h (*lanes 2, 6, and 7*), or 24 h (*lanes 3, 8, and 9*) of cell suspension. Samples (2 μg) were analyzed by Northern blot hybridization with the specific probes to K1, K10, and K14, or 1B15.

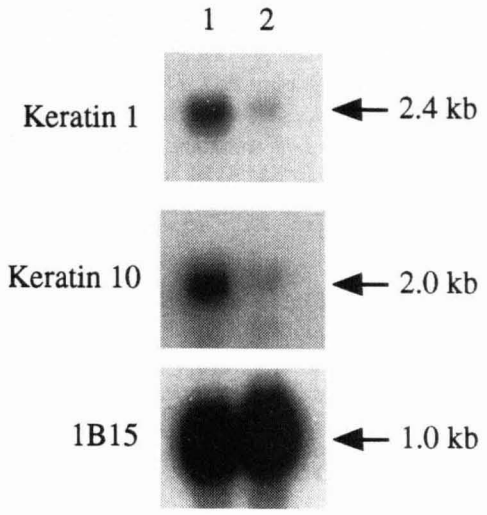

Figure 7. Suprabasal K1 and K10 mRNA expression in subconfluent differentiating cultures. Keratinocytes were plated at 1×10^3 cells/cm^2 (*lane 1*) or 5×10^3 cells/cm^2 (*lane 2*) and grown in complete medium for 2 d, then switched for 3 d to standard medium with 1.5 mM Ca^{++}. Poly(A)RNA was harvested and 5 μg samples analyzed by Northern blot hybridization with the specific probes to K1, K10, or K14, or 1B15.

mitotic cells (data not shown). We predicted that expression of K1 and K10 mRNA would be enhanced by culture of keratinocytes at the lower compared with the higher cell densities, in subconfluent cultures switched to standard, high-calcium medium. The results of this experiment are presented in **Fig 7**. Cells were plated at low density (**Fig 7**, *lane 1*) or higher density (**Fig 7**, *lane 2*) and switched 2 d later to standard medium containing 1.5 mM calcium. K1 and K10 mRNA expression was significantly enhanced at the lower cell density.

Despite the more marked induction of K1 and K10 mRNAs in the low- versus high-density culture conditions, clonal assays demonstrated essentially identical proliferative potential for both cell-density conditions (data not shown). This finding indicates the following: 1) that the irreversible growth arrest induced by cell confluence is not duplicated by increasing the medium calcium concentration, even though both of these culture conditions induced the expression of differentiation-specific keratin markers; and 2) under certain culture conditions, keratinocytes can be induced to express K1 and K10 genes yet retain their full clonogenic potential.

DISCUSSION

Establishment of keratinocyte cultures has provided a useful model to examine cellular control of growth and differentiation [5,8,28,29]. These investigations in human keratinocytes are the first to examine expression and regulation of K1 and K10 genes under fully defined (standard) medium conditions at subconfluent or confluent cell densities. Several factors have been reported to repress or modulate differentiated keratin gene expression, including vitamin A, serum, medium calcium concentrations, and growth factors such as EGF [8,17,30]. Some or all of these factors are present routinely in many culture-medium formulations and likely significantly obscure or modify the intrinsic biologic property of keratinocytes in culture to self-regulate induction of growth arrest and expression of the differentiation program as they recapitulate their *in vivo* function within the epidermis.

Rapidly growing, basal-like human keratinocytes maintain a high degree of clonogenic potential during cultivation at subconfluent cell density regardless of the presence or absence of exogenous growth factors (EGF, insulin and bovine pituitary extract) at low (0.1 mM) or high (1.5 mM) medium concentrations of calcium. However, once cell confluence in culture is attained, clonogenic potential of the keratinocytes decreases rapidly and dramatically. Closer examination of the clonal growth experiments also reveals that a small population of clonogenic keratinocytes exists in cultures that have reached confluence **(Fig 1)**. Small differences in the number and size of keratinocyte clones among the confluent culture conditions are variable (unpublished data). Nonetheless, the small but distinct clonogenic populations contained within these confluent cultures may represent a commitment-resistant population of progenitor keratinocytes that maintain clonogenic potential while the majority of cells become committed, as defined by the loss of their ability to generate clones. These keratinocytes arrest growth irreversibly and have been shown to express eventually several markers of differentiation, including involucrin and cornified envelopes [5,6,20].

Functional evidence of clonogenicity was correlated with molecular markers of programmed differentiation in keratinocytes. We demonstrate that keratinocytes rapidly induce expression of both K1 and K10 mRNAs as cells attain confluence in culture and as clonogenic potential drastically decreases. However, subconfluent cultures propagated in medium containing 0.1 mM Ca^{++} and no exogenous growth factors or containing growth factors and 1.5 mM Ca^{++} do not express K1 or K10 transcripts. Development of intimate cell-cell contacts and probably cell-matrix or other cellular interactions associated with keratinocyte confluence in monolayer culture, even at low (0.1 mM) medium calcium concentration, serves as a crucial signal for keratinocytes to initiate the program of differentiation. The addition of exogenous growth factors, particularly EGF, to the culture medium markedly suppresses steady-state levels of K1 and K10 transcripts; this observation extends on the findings reported for differentiated keratin proteins [31].

Previous studies examining human and mouse keratinocytes have demonstrated that elevating the extracellular calcium concentration induces or enhances expression of differentiation-specific keratins [17,30]. A tissue-specific and calcium-inducible DNA-regulatory region of 4.3 kb located immediately 3′ to the human K1 gene recently has been partially characterized [31]. These enhancer elements identified for the calcium response may be acting indirectly through transcription factors that are induced or activated by elevation of the medium calcium concentration. In the defined culture system reported herein, further activation of these transcription factors by calcium may not be required to initiate expression of K1 once confluence is reached. Alternatively, keratinocytes that attain confluence under low medium calcium concentration may be induced to sustain increases in intracellular concentrations of calcium that could, directly or indirectly, induce transcription of K1 [32]. We have shown previously that rapidly growing, subconfluent keratinocyte cultures propagated with exogenous growth factors efficiently maintain a calcium gradient and prevent fluxes of intracellular calcium that could induce K1 expression and terminal differentiation [33].

Suspension culture, even at high calcium concentration, failed to induce K1 and K10 mRNAs in uncommitted, rapidly growing keratinocytes. Addition of EGF to suspension cultures decreased steady-state levels of K1 and K10 transcripts. This finding correlates well with the suppressive effect of EGF on K1 and K10 mRNA levels in adherent, confluent keratinocytes cultivated in the absence of exogenous growth factors at either low or high medium calcium concentration.

Recently, Drozdoff and Pledger [25] reported that murine keratinocytes placed in suspension culture express the K1 and K10 gene products regardless of the medium concentration of calcium. However, keratinocytes were grown to confluence before placement in suspension culture and therefore already were induced to express K1 mRNA. We extend their findings to show that cell confluency is a critical event in the induction of differentiation-specific keratin genes. We also demonstrate that under selected culture conditions, namely subconfluent cultures grown at low cell density in defined medium containing high calcium concentration (1.5 mM), expression of K1 and K10 mRNA is induced significantly compared to cells grown at higher cell density. Under certain conditions, elevation of medium calcium concentration promotes K1 and K10 transcript expression. However, regardless of suprabasal keratin gene expression, the clonogenicity of low– or higher–cell-density subconfluent cultures is equivalent. Therefore, under some growth-arrest states, keratinocytes are able to express differ-

entiation markers such as K1 and K10 without losing their clonogenic potential.

Induction of K1 and K10 expression by keratinocytes reaching confluence has physiologic relevance. Normal, unperturbed epidermis can be regarded as a "confluent" tissue, and suprabasal keratins are continually expressed as proliferative keratinocytes become post-mitotic (irreversibly growth arrested) and committed to terminal differentiation. However, when the epidermis is disrupted by injury, cellular events are activated to initiate reepithelialization. This *in vivo* state may be mimicked by subconfluent culture. Under these similar conditions *in vitro* and *in vivo*, expression of K1 and K10 is suppressed. In addition, epidermal hyperproliferative diseases such as psoriasis—with marked overexpression of EGF-related growth factors such as transforming growth factor-α, amphiregulin, and heparin-binding EGF, and with delayed expression of K1 and K10—may be represented in these studies by confluent cultures to which exogenous EGF has been added and in which K1 and K10 gene expression is suppressed [34–36].

Keratinocytes exhibit the capacity to regulate their own clonogenic potential and differentiation-specific keratin gene expression in culture. Attaining cell confluence is a major, and perhaps the primary, intrinsic biologic mechanism by which keratinocytes lose clonogenicity, irreversibly arrest growth, and express the suprabasal keratins K1 and K10. It will be crucial to identify the molecular trigger(s) that is activated with cell confluence to advance our understanding of the cell biology of commitment and terminal differentiation of the keratinocyte.

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